

5 *Xba*I/*Sal*I (filled in with Klenow) linearized pRS416 CYC1
(Mumberg, et al., *Gene* **156**:119-122 (1995)).

MB1644 and MB2478 are *URA3*-based *S. cerevisiae* expression plasmids that contain the wild-type *lovE* gene. They are both derivatives of MB1199. MB1199 was created 10 by using primers MO841 (SEQ ID NO:11) and MO842 (SEQ ID NO:12) to amplify the *lovE* ORF from *A. terreus* cDNA. Gateway (Invitrogen™, Carlsbad, CA) Cloning Technology (US Patent 5,888,732) was used to clone the *lovE* PCR fragment into the gateway entry vector pDONR206 (Invitrogen™, 15 Carlsbad, CA) to create MB1199. Similarly, Gateway Cloning Technology was used to transfer the *lovE* ORF from MB1199 into MB968 to create MB2478 and into MB969 (U.S. Serial No. 60/198,335, filed April 18, 2000) to create MB1644.

20 MB2848 is a derivative of MB968 that contains a *lovE*-*AT274* chimera. The *lovE* portion of MB2848 was derived by using oligos MO841 (SEQ ID NO:11) and MO2278 (SEQ ID NO:13) to PCR amplify the *lovE* DNA binding domain from *A. terreus* cDNA. A second round of PCR was performed with 25 primers MO343 (SEQ ID NO:14) and MO2278 to add appropriate Gateway Cloning Technology compatible sequences. The *At274* portion of MB2848 can be derived by using primers MO2273 (SEQ ID NO:15) and MO2274 (SEQ ID NO:16) to PCR amplify the carboxy-terminal domain of *At274* from *A. terreus* cDNA. A second round of PCR was performed with 30 primers MO344 (SEQ ID NO:17) and MO2273 to add appropriate Gateway Cloning Technology compatible sequences. The *lovE* and *At274* PCR products were cut with *Bam*HI and purified over a QIAquick PCR purification kit (Qiagen, Valencia, 35 CA) according to manufacturer's instructions. Finally, the products were mixed 3-4 hours in a standard ligation reaction and used in Gateway entry and destination reactions to create MB2848.

40 Gateway cloning technology was used to clone the *lovE* variants of interest into plasmid MB1419 which is a filamentous fungal expression vector. The MB1419 fungal selection marker is the *A. nidulans* *GPD* promoter controlling the *ble* gene from *S. hindustanus*. The

5 transgene is controlled by the *A. nidulans PGK* promoter.
A. terreus strain MF117 is a derivative of *A. terreus*
strain ATCC 20542.

Example 2: PCR Mutagenesis of the *lovE* DNA Binding Domain

10 The zinc finger DNA binding domain of *lovE* is encoded by nucleotides 100-201 (SEQ ID NO:92). Oligos MO2624 (SEQ ID NO:18) and MO2654 (SEQ ID NO:19) were used to PCR amplify a *lovE* containing fragment from plasmid MB2478. The 1.7 kb product contains nucleotides 212-1410 of *lovE* and ~500
15 bp of flanking vector sequence. Two rounds of standard PCR (1.5 mM MgCl₂) were performed with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, Ca) according to the manufacturer's instructions.

Plasmid MB2478 was cut with *KpnI-BamHI* to release a 1.1
20 kb fragment containing the At274 portion of the *lovE*-At274 chimeric open reading frame. The remaining 5.5 kb vector sequence retains the *lovE* DNA binding domain.

Example 3: PCR Mutagenesis of the *lovE* Open Reading Frame

25 *lovE* open reading frame insert was prepared according to the following procedure. Oligo pairs MO2680 (SEQ ID NO:20) /MO2686 (SEQ ID NO:21), MO2681 (SEQ ID NO:22) /MO2686, and MO2700 (SEQ ID NO:23) /MO2701 (SEQ ID NO:24) were used to PCR amplify the entire *lovE* open reading
30 frame from plasmid MB2478. The PCR products differ in the amount of 5' and 3' vector sequence flanking the *lovE* open reading frame.

PCR was performed using a GeneMorph PCR mutagenesis kit (Stratagene, La Jolla, Ca) according to manufacturer's
35 instructions to achieve medium and high range mutation frequencies.

Plasmid MB2478 was cut with *Asp718/XbaI* to release a 1.7 kb fragment. The remaining 5.0 kb vector sequence completely lacks *lovE* ORF sequence.

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Example 4: Transformation and Selection for G418R Isolates

5 All PCR products were purified using a QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions. All vectors were gel purified using a QIAquick gel extraction kit (Qiagen) according to manufacturer's instructions.

10 The mutagenesis strategy of Muhlrad *et al.* (*Yeast* 8:79-82 (1992)) was used which involves cotransforming a mutated PCR product and gapped plasmids into *S. cerevisiae*, and then screening for *in vivo* recombinants having the desired phenotype).

15 Transformation of *Saccharomyces cerevisiae* was accomplished by the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) protocol (Woods R.A. and Gietz R.D. *Methods Mol. Biol.* 177:85-97 (2001)) with a 1:5 molar ratio of vector:insert DNA to 20 generate >55,000 *in vivo* recombinant transformants on SC-Ura plates. Transformants were transferred by replica printing to YPD plates containing 100 µg/ml G418 and allowed to grow for 2-4 days at 30°C (Figure 1).

25 Drug resistant clones were confirmed in secondary assays including growth on G418 concentrations up to 2000 µg/ml. The plasmid-dependence of the phenotype was determined by observing the re-appearance of drug sensitivity correlating with loss of the library plasmid. 30 *lovE* variant plasmids were recovered from promising candidates (Hoffman and Winston (1986) *Gene* 57:267). More than 70 *lovE* variants were identified and definitively characterized by DNA sequence and/or restriction digestion analysis.

35 Table 3 summarizes the G418 resistance phenotype and sequence analysis of 26 of these variants.